

# Identification of a new intermediate state that binds but not activates transducin in the bleaching process of bovine rhodopsin

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**Abstract** Using time-resolved low-temperature spectroscopy, we have examined whether or not bovine rhodopsin has a unique transducin-binding state, meta I<sub>b</sub>, previously detected from chicken rhodopsin. Unlike chicken meta I<sub>b</sub>, bovine meta I<sub>b</sub> was detected only by detailed kinetics analysis of the bleaching process, but it was stabilized by transducin and visualized in the observed spectral changes. From the effect of GTPγS, it was revealed that meta I<sub>b</sub> induced no GDP-GTP exchange reaction in transducin. Thus meta I<sub>b</sub> is a common intermediate of vertebrate rhodopsin and transducin is activated in two steps by meta I<sub>b</sub> and meta II.

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**Key words:** Rhodopsin; Transducin; Photobleaching process; Activation process

## 1. Introduction

Rhodopsin, the visual pigment present in rod photoreceptor cells, is a prototypical G protein-coupled receptor that receives a light signal from the outer environment using a light absorbing chromophore, 11-*cis*-retinal. In contrast to a large variety of receptors which are activated by diffusible agonist ligands, rhodopsin is synchronously activated by light. The chromophore acts as an intrinsic spectroscopic probe to monitor the protein structural changes so that each step of the changes in rhodopsin has been identified as an intermediate state having specific absorption spectrum [1–4]. The state that activates retinal G protein transducin has been also investigated using bovine rhodopsin as a sample and it was thought that one of the intermediates, metarhodopsin II (meta II), is responsible for the transducin activation [5–7].

Recently we have developed a new technique called time-resolved low-temperature spectroscopy to investigate the detailed thermal reactions of photoactivated rod (rhodopsin) and cone visual pigments of chicken retinas for elucidating the functional difference between rod and cone photoreceptor cells [8–10]. During the course of studies, we have found that chicken rhodopsin has a new intermediate state having absorption maximum at 460 nm which appears in between formally identified metarhodopsin I (meta I, now referred to as

meta I<sub>a</sub>) and meta II. The intermediate named meta I<sub>b</sub> can form a complex with transducin but induce no GDP-GTP exchange reaction in transducin, while the exchange reaction occurs in the meta II-transducin complex. Thus we suggested that the activation process of transducin by chicken rhodopsin is separated in two steps: one is the process of complex formation and the other is that of the enzymatic reaction (GDP-GTP exchange reaction) [11].

Although photochemical and subsequent thermal reactions of bovine rhodopsin have been extensively studied [12–14], there is no report detecting a meta I<sub>b</sub>-like intermediate. Since the manner of interaction with transducin is different between meta I<sub>b</sub> and meta II, the identification of meta I<sub>b</sub> from bovine rhodopsin is important for generalization of the two-step activation mechanism of transducin by rhodopsin. Furthermore, bovine rhodopsin is the most popular visual pigment which has been subjected to the biochemical and molecular biological investigations and it is the potential candidate to elucidate the physiological significance of meta I<sub>b</sub> at submolecular resolution. Thus we have examined whether or not the meta I<sub>b</sub> is present in the bleaching process of bovine rhodopsin and it behaves like that of chicken rhodopsin. Current findings clearly showed that meta I<sub>b</sub> is really present in the bleaching process of bovine rhodopsin, but its detection is very hard, if transducin is not present in the rhodopsin sample.

## 2. Materials and methods

### 2.1. Preparation of bovine rhodopsin and transducin

Rhodopsin was extracted from ROS of bovine retinas using CHAPS as a detergent and purified by means of ConA column chromatography according to the method described previously [15]. Briefly, rhodopsin extracted with buffer A (50 mM HEPES, 1% (w/v) CHAPS, 140 mM NaCl, 1 mM MnCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.1 mM PMSF, 4 μg/ml leupeptin, 50 KIU/ml aprotinin, 1 mM DTT, pH 7.5 at 4°C) was applied to a ConA Sepharose (Pharmacia) column, from which rhodopsin was eluted with buffer B (50 mM HEPES, 0.6% (w/v) CHAPS, 0.8 mg/ml PC, 140 mM NaCl, 1 mM MnCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.1 mM PMSF, 4 μg/ml leupeptin, 50 KIU/ml aprotinin, 1 mM DTT, pH 7.5 at 4°C) supplemented with 0.2 M methyl α-D-mannopyranoside. The concentration of rhodopsin in the sample was estimated by its absorbance at the maximum (molar extinction coefficient = 41 200, [16]). All the procedures were performed at 4°C and under dim red light. Bovine transducin was purified from fresh bovine retinas according to the method previously described [11,17].

### 2.2. Time-resolved low-temperature spectroscopy

To investigate the effects of transducin and GTPγS on the bleaching process of rhodopsin, the following three samples in buffer C (50 mM HEPES, 0.6% (w/v) CHAPS, 0.8 mg/ml PC, 140 mM NaCl, 1.6 mM MgCl<sub>2</sub>, 0.2 mM MnCl<sub>2</sub>, 0.2 mM CaCl<sub>2</sub>, 0.1 mM PMSF, 4 μg/ml leupeptin, 50 KIU/ml aprotinin, 1 mM DTT, 0.2 M methyl α-D-mannopyranoside, 20% (w/v) glycerol, pH 7.5 at 4°C) supplemented with equal volume of glycerol were prepared: sample A contained 3.90 μM rhodopsin, sample B contained 3.90 μM rhodopsin and 10.1 μM

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**Abbreviations:** GTPγS, guanosine 5'-O-(3-thiotriphosphate); ROS, rod outer segment(s); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ConA, concanavalin A; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; PC, L-α-phosphatidylcholine form egg yolk

transducin, and sample C contained 3.90  $\mu\text{M}$  rhodopsin, 10.1  $\mu\text{M}$  transducin, and 421  $\mu\text{M}$  GTP $\gamma\text{S}$ .

Absorption spectra were recorded on a Shimadzu MPS-2000 recording spectrophotometer interfaced to a personal computer (NEC PC9801RA). An optical cryostat (Oxford, CF1204) with an optical cell of 1-cm light path was used to record the spectral changes at low temperatures. The sample temperature was regulated to within 0.1°C by a temperature controller (Oxford, ITC4) attached to the cryostat. The sample was irradiated with light from a 1-kW tungsten-halogen lamp (Rikagaku-Seiki). The wavelength of the irradiation light was selected with a glass cutoff filter (Toshiba). A 5-cm water layer was placed in front of the light source to remove heat from the irradiation light. Thermal reactions of intermediates initiated by irradiation of the rhodopsin sample at low temperature were monitored by recording absorption spectra with intervals of 2–30 min until the reactions were almost saturated. The recording of each spectrum in the wavelength region from 650 to 330 nm required 42 s.

The amount of rhodopsin photoconverted to the intermediates by the irradiation at low temperature was estimated by the method previously described [11].

### 2.3. Spectral analyses

The spectral changes due to the thermal reactions of intermediates were analyzed by SVD (singular value decomposition), one of the mathematical methods using the matrix transformation procedures [18], to estimate the number of spectrally distinct intermediates that are necessary for describing the spectral changes. Global exponential fitting [14,19] was then applied to calculate the apparent rate constant of each reaction and its spectral component called b-spectrum. For example, from the spectral changes shown in the middle panel of Fig. 1a, two sets of apparent rate constants and b-spectra (lower panel of Fig. 1a) were calculated. This indicated that three intermediate states were needed to reproduce the spectral changes. It should be noted that a spectral shape of b-spectrum depends on the absorption spectra of the intermediates and/or the rate constants for the transitions among intermediates [14]. These calculations were performed by a program written on a software Igor Pro (WaveMetrics, Inc.) of Macintosh Performa 5210 (Apple Computer). Because of easy comparison with the observed spectral changes shown in Figs. 1–3, the opposite signed b-spectra are shown in Figs. 1 and 4.

## 3. Results and discussion

### 3.1. Identification of a meta $I_b$ -like intermediate in the photobleaching process of bovine rhodopsin

To examine whether or not meta  $I_b$  is present in the photobleaching process of bovine rhodopsin, the thermal reactions of intermediates produced by irradiation of bovine rhodopsin were investigated by means of time-resolved low-temperature spectroscopy and compared with those observed in chicken rhodopsin (Fig. 1). In each experiment, the sample was cooled to  $-20$  or  $-25^\circ\text{C}$  and irradiated with a  $>570\text{-nm}$  light for 30 s, followed by recording the spectral changes due to the thermal reactions of intermediates. The spectral changes are expanded in the middle panels of each figure, in which the changes are represented as the difference spectra calculated by subtracting the spectrum recorded immediately after the irradiation from those recorded at later times after the irradiation. It should be noted that the difference in temperature between the two experiments is due to the difference in thermal stability between respective meta II [8,9].

The spectral changes observed in chicken rhodopsin were already analyzed by Tachibanaki et al. [11]. The irradiation caused a formation of a mixture containing mainly meta  $I_a$  (curve 2 in the upper panel of Fig. 1a), and subsequent incubation resulted in conversion from meta  $I_a$  to meta II via meta  $I_b$  (middle panel of Fig. 1a, curves 2'–12'). The presence of three intermediates in the conversion process was confirmed by SVD and global fitting analysis (lower panel of

Fig. 1a). Namely, the spectral changes are expressed by two b-spectra; the former (lower panel of Fig. 1a, upper curve) reflects the conversion process of meta  $I_a$  to meta  $I_b$ , and the latter (lower panel of Fig. 1a, lower solid curve) that of meta  $I_b$  to meta II. The shift of the intersection point (middle panel of Fig. 1a) is also one of the indications that the process involves more than two intermediates.

The spectral changes observed in bovine rhodopsin (middle panel of Fig. 1b, 2'–12') is rather different from those observed in chicken rhodopsin. The shift of the intersection point is less pronounced than that in chicken rhodopsin (mid-

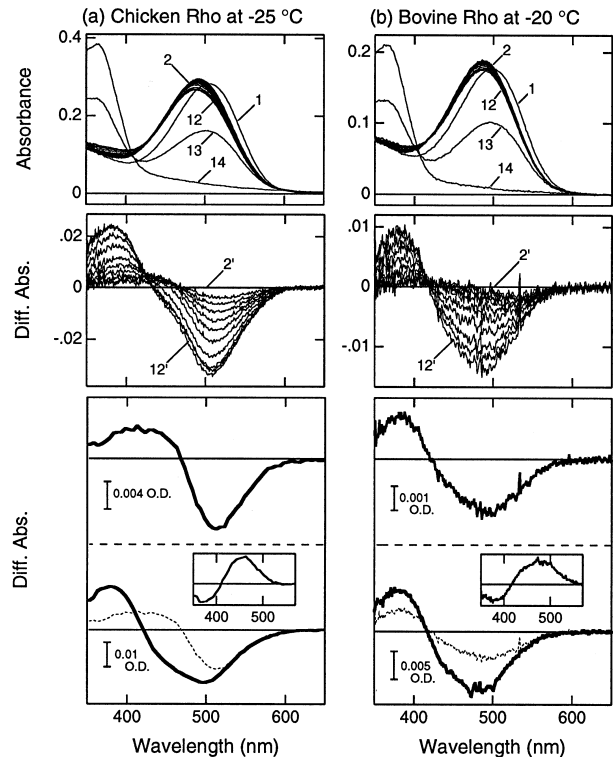


Fig. 1. Thermal reactions of intermediates produced by irradiation of chicken (a) and bovine (b) rhodopsins at  $-25$  and  $-20^\circ\text{C}$ , respectively. Upper panels: chicken (a) and bovine (b) rhodopsins were cooled to  $-25$  and  $-20^\circ\text{C}$  (curves 1), respectively and irradiated with  $>570\text{-nm}$  light for 30 s (curves 2), followed by incubation at these temperatures for 2.5, 5, 10, 20, 40, 80, 160, 320, 640, and 1280 min (curves 3–12 in a), and 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 min (curves 3–12 in b), respectively. Then the samples were warmed to  $20^\circ\text{C}$  and 1 M hydroxylamine was added to the samples at a final concentration of 10 mM. After recording the spectra at  $-25$  and  $-20^\circ\text{C}$  (curves 13), they were irradiated with  $>500\text{-nm}$  light for  $>20$  min at  $0^\circ\text{C}$  and the spectra were recorded at  $-25$  and  $-20^\circ\text{C}$  (curves 14), respectively. Middle panels: difference spectra due to the thermal reactions of intermediates of chicken (a) and bovine (b) rhodopsins are calculated by subtracting the spectrum recorded immediately after the irradiation (curves 2 in upper panels) from the spectra recorded at later times after the irradiation (curves 3–12 in upper panels), respectively. Lower panels: b-spectra calculated from the difference spectra shown in the middle panels. The time constants for the first (upper curve) and second (lower solid curve) b-spectra of chicken rhodopsin sample are 19.4 min and 184.9 min, respectively. The time constants of bovine rhodopsin sample are 18.2 and 201.6 min, respectively. To make clear the difference in shape between first and second b-spectra, the first b-spectra (dotted curves) were superimposed on the respective second b-spectra after normalizing their absorbances at  $>560$  nm, which reflected the spectral components of meta  $I_a$ . Inset: difference spectra between meta  $I_b$  and meta II calculated from the b-spectra.

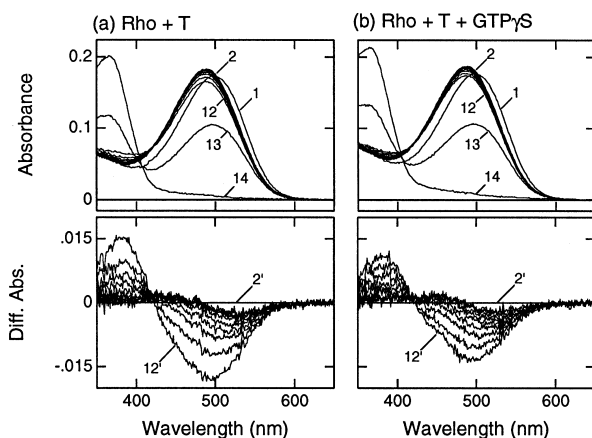


Fig. 2. Effects of transducin and/or GTP $\gamma$ S on the thermal reactions of bovine rhodopsin intermediates. Upper panels: the samples containing rhodopsin+transducin (sample B) (a) and that containing rhodopsin+transducin+GTP $\gamma$ S (sample C) (b) were cooled to  $-20^{\circ}\text{C}$  (curves 1) and irradiated with  $>570\text{-nm}$  light for 30 s (curves 2), followed by incubation at this temperature for 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 min (curves 3–12). Then the samples were warmed to  $20^{\circ}\text{C}$  and 1 M hydroxylamine was added to the samples at a final concentration of 10 mM. After recording the spectra at  $-20^{\circ}\text{C}$  (curves 13), they were irradiated with  $>500\text{-nm}$  light for  $>20$  min at  $0^{\circ}\text{C}$  and the spectra were measured at  $-20^{\circ}\text{C}$  (curves 14). Lower panels: difference spectra obtained by subtracting the spectrum recorded immediately after the irradiation (curves 2 in upper panels) from the spectra recorded at later times after the irradiation (curves 3–12 in upper panels) are presented.

dle panel of Fig. 1a). The final spectrum in bovine rhodopsin (curve 12' in the middle panel of Fig. 1b) seems to represent the difference spectrum between two intermediates, one is meta  $I_a$  having maximum at about 490 nm and the other is meta II having maximum at about 380 nm. This is in contrast with that the final spectrum in chicken rhodopsin (curve 12' in the middle panel of Fig. 1a) exhibits small negative absorbances around 460 nm due to the presence of a considerable amount of 460 nm pigment (meta  $I_b$ ) in addition to meta  $I_a$  and meta II. These facts might suggest that the bleaching process of bovine rhodopsin is different from that of chicken rhodopsin and there is present no intermediate in between meta  $I_a$  and meta II of bovine rhodopsin. However, SVD analysis showed that the spectral changes observed in bovine rhodopsin are also expressed by two b-spectra (lower panel of Fig. 1b), suggesting the presence of another intermediate in between meta  $I_a$  and meta II. Important point is that the first b-spectrum exhibits an intersection point and a negative maximum about 10 nm red-shifted from those of the second b-spectrum (lower curves in lower panel of Fig. 1b), although the difference in shape between two b-spectra are not so prominent. Thus the spectra of intermediates appearing in the process are different among each other. Hereafter the intermediate other than meta  $I_a$  and meta II is referred to as meta  $I_b$  of bovine rhodopsin.

The first b-spectrum in bovine rhodopsin is considerably different in shape from that in chicken rhodopsin, while the second b-spectra are similar with each other (lower panels of Fig. 1a and b). Since the spectra of bovine and chicken meta  $I_a$  and meta II are similar with each other, respectively, the difference in first b-spectrum is due to the difference in spectrum of meta  $I_b$  and/or that in rate constants for the transitions among intermediates between bovine and chicken rho-

dopsin systems. Thus it is important to estimate the absorption spectrum of meta  $I_b$  of bovine rhodopsin to further elucidate the difference. The calculation procedure was already described in the previous paper [11], in which difference spectrum between meta  $I_b$  and meta II in chicken rhodopsin (inset in lower panel of Fig. 1a) was obtained. The results clearly showed that the meta  $I_b$  of bovine rhodopsin (inset in lower panel of Fig. 1b) exhibits a spectrum similar to that of meta  $I_b$  of chicken rhodopsin, although the extent of meta  $I_b$  appearing in the process is very low. Thus we concluded that bovine rhodopsin has meta  $I_b$  spectrally similar to that of chicken rhodopsin, and the difference in shape of the first b-spectrum is mainly due to the differences in rate constants between them. It should be noted that meta  $I_b$  has an absorption maximum (470 nm) considerably red-shifted from that of meta II ( $\lambda_{\text{max}} = 380\text{ nm}$ ), suggesting that it has a protonated retinylidene Schiff base as its chromophore.

### 3.2. Interaction states of bovine rhodopsin with transducin

Since the analysis of bleaching process of bovine rhodopsin indicated the presence of meta  $I_b$ , we have further investigated whether or not it can interact with transducin in a manner similar to that of meta  $I_b$  of chicken rhodopsin. For this purpose, we compared thermal reactions of meta  $I_b$  in the sample containing only rhodopsin with that containing rhodopsin and transducin. We also investigated whether or not GTP $\gamma$ S can affect the interaction between meta  $I_b$  and transducin.

Fig. 2 shows spectral changes recorded after irradiation of bovine rhodopsin at  $-20^{\circ}\text{C}$  in the sample containing trans-

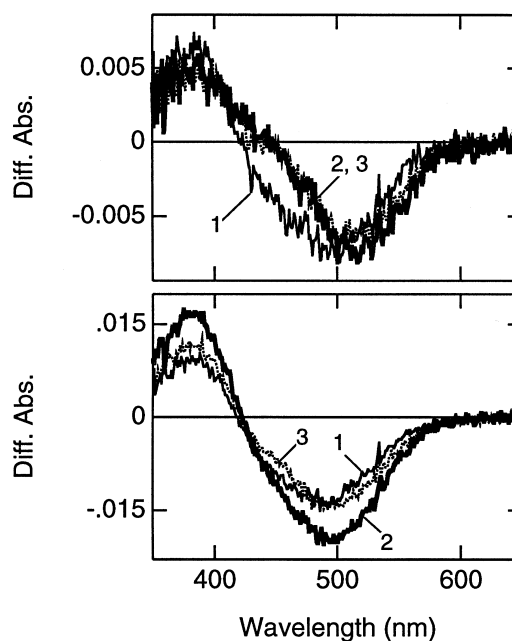


Fig. 3. Effects of transducin and/or GTP $\gamma$ S on the thermal reactions of meta  $I_b$  and meta II of bovine rhodopsin. Curves 1 (thin), 2 (thick) and 3 (dotted) are the spectra obtained from the samples containing rhodopsin (sample A), rhodopsin+transducin (sample B), and rhodopsin+transducin+GTP $\gamma$ S (sample C), respectively. These curves are calculated by subtracting the spectra immediately after irradiation of the samples from those recorded at 128 min (upper panel) or 1024 min (lower panel) after the irradiation. The spectra are normalized so that they represent the changes induced by the photoreaction of 0.075 absorbance of rhodopsin.

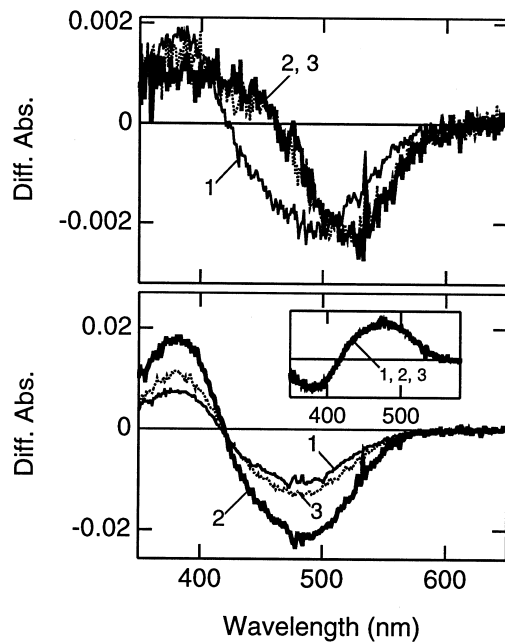


Fig. 4. B-spectra calculated from the spectral changes observed at  $-20^{\circ}\text{C}$ . Upper and lower curves represent the first and second b-spectra. B-spectra of the samples containing rhodopsin (sample A), rhodopsin+transducin (sample B), and rhodopsin+transducin+GTP $\gamma$ S (sample C) are represented by curves 1 (thin), 2 (thick) and 3 (dotted), respectively. The time constants for the first b-spectra of samples A, B, and C are 18.2, 29.1, and 22.3 min, respectively. Those for the second b-spectra of samples A, B, and C are 201.6, 709.5, and 426.4 min, respectively. The spectra are normalized so that they represent the changes induced by the photoreaction of 0.075 absorbance of rhodopsin. Inset: normalized difference spectra between meta  $I_b$  and meta II calculated from the b-spectra.

ducin (sample B) or that containing both transducin and GTP $\gamma$ S (sample C). As compared with the spectral changes observed in the sample containing only rhodopsin (sample A, upper and middle panels of Fig. 1b), the shift of intersection point became prominent. Furthermore, superposition of the spectra recorded at 128 min after the irradiation (upper panel of Fig. 3) clearly shows the increase in absorbance around 470 nm due to the accumulation of meta  $I_b$  in samples B and C. Thus meta  $I_b$  is stabilized by transducin. It should be noted that the spectrum observed in sample C is identical in shape with that in sample B, indicating that GTP $\gamma$ S did not abolish the stabilization effect of transducin on meta  $I_b$ . These results strongly suggest that meta  $I_b$  induces no GDP-GTP exchange reaction in transducin.

The effect of transducin on the thermal reaction of meta II is also evident in Fig. 3 (lower panel), in which the spectra recorded at 1024 min after the irradiation are superposed. Namely, addition of transducin increased the absorbance at 380 nm due to the accumulation of meta II (curve 2 in Fig. 3, lower panel), while GTP $\gamma$ S abolished the increase (curve 3 in Fig. 3, lower panel). Thus, transducin can stabilize meta II and GTP $\gamma$ S can abolish the interaction between meta II and transducin.

It should be emphasized that the effects of transducin and/or GTP $\gamma$ S on the thermal reactions of meta  $I_b$  and meta II of bovine rhodopsin were clearly observed in the raw data of spectral changes. This is in contrast to the case of chicken rhodopsin in which binding of meta  $I_b$  to transducin was reasonably detected only by the analyses of SVD and global

fitting of the spectral data [11]. However, the above interpretations based on the spectra recorded at selected times after the irradiations should be confirmed by the detailed analysis of the spectral data. SVD and global fitting analyses clearly showed that the spectral changes observed in the samples B and C were also expressed by two b-spectra (Fig. 4), which reflect the conversion from meta  $I_a$  to meta  $I_b$  and that from meta  $I_b$  to meta II, respectively. These results indicate that the number of intermediates appearing in the conversion process did not change even in the presence of transducin and GTP $\gamma$ S. Comparison of the first b-spectra shows the increases in absorbance around 470 nm due to the accumulation of meta  $I_b$  in the samples B and C (upper panel of Fig. 4), while accumulation of meta II as evidenced by the increase in absorbance at 380 nm was observed only in the second b-spectrum of sample B (lower panel of Fig. 4). Therefore, it is confirmed that meta  $I_b$  binds to transducin and meta II induces GDP-GTP exchange reaction in transducin. Furthermore, the difference absorption spectra between meta  $I_b$  and meta II calculated based on the b-spectra were identical in shape among three samples (lower panel of Fig. 4, inset), indicating that the differences in shape of b-spectra are due to the differences in rate constants of the intermediates among three samples. Taken together, we concluded that bovine rhodopsin activates transducin in a manner similar to that of chicken rhodopsin: meta  $I_b$  only binds to transducin, while meta II activates transducin.

Identification of the new transducin-binding state in bovine rhodopsin would give a new insight on the activation mechanism of transducin by comparing with the results obtained from the mutational experiments of bovine rhodopsin. First, the intermediate observed in the rhodopsin mutant, E113A/A117E, having a counterion one helical turn up to the original rhodopsin exhibits a red-shifted spectrum like meta  $I_b$  and activates transducin [20]. Present study confirmed that the binding to transducin really occurs in the red-shifted intermediate and emphasized that the deprotonation of the Schiff base chromophore may be a secondary event during the course of conformational changes which results in activating transducin. Second, rhodopsin mutants that bind but fail to activate transducin were prepared by deletions or replacements of the specific regions of the cytoplasmic loops of rhodopsin [21,22]. Since binding to transducin was monitored as an increased amount of meta II or an increased scattering of the sample in these experiments, direct comparison between these results and those obtained in the present study are difficult. However, if one can assume that the binding sites of meta II to transducin involve those of meta  $I_b$ , meta  $I_b$  would bind to transducin by the half of the second cytoplasmic loop. Thus further characterization of the meta  $I_b$ -transducin complex will give the information of the stepwise changes in the cytoplasmic loops of rhodopsin which couple with the conformational changes of transducin.

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